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Decreased protein C, protein S, and antithrombin levels are predictive of poor outcome in Gram-negative sepsis caused by *Burkholderia pseudomallei*☆

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Summary

Background: Acute septicemic melioidosis is associated with systemic release of endotoxin and the proinflammatory cytokines tumor necrosis factor (TNF)-alpha, interleukin-1, and interleukin-6. Excessive release of these cytokines may lead to endothelial injury, depletion of naturally occurring endothelial modulators, microvascular thrombosis, organ failure, and death.
Method: Plasma samples drawn at baseline and after initial antimicrobial therapy in 30 patients with suspected acute severe melioidosis were assayed for D-dimer levels, protein C and protein S antigen levels, and antithrombin functional activities.

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Results: Both baseline and continued deficiencies of protein C, protein S, and antithrombin were statistically associated with a poor outcome by logistic regression. Baseline D-dimer levels were significantly higher in fatal cases than survivors and correlated inversely with protein C and antithrombin, suggesting both increased fibrin deposition and fibrinolysis.
Conclusion: The inflammatory response to systemic *Burkholderia pseudomallei* infection leads to depletion of the natural endothelial modulators protein C, protein S, and antithrombin. Both baseline and continued deficiency of these endothelial modulators is predictive of poor outcome in melioidosis.

Introduction

Melioidosis is an infection caused by the organism *Burkholderia pseudomallei*, a small, motile, Gram-negative, non-spore-forming obligate aerobe found in soil and surface water, e.g., rice paddies in endemic areas.[1] Infection with this organism can occur by direct inoculation, inhalation, aspiration, or ingestion.[2] The clinical presentation of melioidosis includes fever of unknown etiology, localized skin/soft tissue abscess, pneumonia, and septicemia.[3] In Northeast Thailand, melioidosis is the most common cause of septicemia during the rainy season.[4] The disease therefore provides an ideal opportunity to study many cases of Gram-negative sepsis caused by a single organism, in a homogeneous population, and at a single study site, thus bypassing many of the problems that beset other sepsis studies.[5]

Sepsis irrespective of microbiologic cause is associated with the interplay of inflammation and coagulation. Experimental studies have shown that endotoxin, as well as the proinflammatory cytokines tumor necrosis factor (TNF)-alpha, interleukin-1 (IL-1), and interleukin-6 (IL-6) can lead to activation of the extrinsic pathway of coagulation by causing the expression of tissue factor on the endothelium and monocytes.[6,7] High circulating levels of endotoxin, TNF and IL-6 have each been demonstrated in melioidosis patients.[8,9]

Three endogenous anticoagulants that regulate the inflammatory and coagulation cascade in sepsis are activated protein C (APC), antithrombin and protein S. Activated protein C is generated from the inactive zymogen protein C in the setting of thrombin production. It inhibits factor V thereby preventing further thrombin generation, restores fibrinolysis by inactivating plasminogen activator inhibitor (PAI-1) and has anti-inflammatory properties.[10–14] Antithrombin is a serine protease inhibitor that inactivates multiple clotting enzymes (Xa, IXa, XIa) and, in particular, thrombin by forming thrombin–antithrombin complexes (TAT).[15] Antithrombin too has distinct anti-inflammatory properties.[16] Protein S is the third endothelial modulator that may be affected in the septic state. Protein S is a vitamin K-dependent protein that has no enzymatic activity of its own. This molecule enhances the activity of APC by inhibiting the protective effect of factor Xa on factor Va, thereby making factor Va more susceptible to cleavage by APC.[17] Severe sepsis can lead to the decreased production, increased consumption and inhibition of these endogenous anticoagulants.[18]

In this study we measured levels of protein C, protein S, and antithrombin in a homogeneous group of patients with suspected acute severe melioidosis, to assess if early and continued deficiency of these endothelial modulators of coagulation and inflammation is predictive of a poor outcome. D-dimer levels were measured to assess if fibrin deposition and lysis were predictive of outcome. Finally, we attempted to correlate levels of these endothelial modulators with initial D-dimer and inflammatory cytokine levels.

Patients and methods

Patients

A subset of samples from patients with suspected acute severe *Burkholderia pseudomallei* infections enrolled in a prospective, randomized treatment trial of high-dose intravenous imipenem versus ceftazidime, conducted at Sappasitprasong Hospital in Northeast Thailand, was randomly selected to evaluate retrospectively biomarker levels of protein C, protein S, antithrombin, and D-dimer in acute severe melioidosis. Ethical approval for the trial was received from the Ethical and Scientific Subcommittee of the Thai Ministry of Public Health. The trial enrolled 296 patients with suspected melioidosis between July 1994 and November 1997. The results indicated no statistical significance in the difference in mortality between the two treatment groups ($p = 0.96$).[19]

Blood sampling procedures

Venous blood samples from patients with acute septicemic melioidosis were collected in heparinized tubes at baseline and at times up to 24 h after starting antimicrobial therapy. Plasma was separated immediately and stored at −70 °C until assays were performed. Before assay, the plasma samples were thawed for 10 min at 37 °C and then placed on ice.

Assay methods

The investigators randomly selected plasma samples from 30 patients with suspected acute septicemic melioidosis to send to Lilly Research Laboratories for analysis of the biomarkers. The Lilly group was blinded to patient outcome.

Protein C antigen

Total protein C antigen levels in the heparinized plasma were measured with a commercially available antigen assay Asserachrom Protein C (Stago Diagnostica, Asnieres, France) following the manufacturer's instructions. This sandwich-based ELISA uses rabbit antihuman protein C F(ab) 2 fragments as the capture antibody. The normal range of protein C antigen in 30 normal plasmas was 73.7—140%.

Protein S antigen

Free protein S antigen levels were measured with a commercially available antigen assay, Asserachrom Protein S (Stago Diagnostica, Asnieres, France) following the manufacturer's instructions. The normal range of free protein S antigen in 30 normal plasmas was 55—116%.

Antithrombin activity

Activity levels were measured with a commercially available assay, Stachrom AT III (Stago Diagnostica, Asnieres, France). The chromogenic activity was measured with the Stago Compact Coagulation Analyzer. The normal range of antithrombin activity established in 30 normal plasmas was 86—124%.

D-dimer

D-dimer levels were quantified by a latex immunoassay method using Liatest D-DI (Diagnostica Stago, Asnieres, France). The increase in absorbance at 540 nm due to binding of D-dimer to the antibody-coated latex beads was measured with the Stago Compact Coagulation Analyzer. The normal range established for this assay was 0.05—0.47 μg/mL.

Cytokine data, demographics, and baseline characteristics

Baseline demographic, clinical and laboratory characteristics of the patients in this study were recorded on admission. IL-6 data have been published previously.[9,19]

Statistical methods

Results of samples drawn during the illness were averaged for each patient. Assay data were analyzed with patient demographic information and outcomes. ANOVA analyses were performed to compare baseline values of the biomarkers protein C antigen, protein S antigen, antithrombin functional activity, and D-dimer in survivors and fatal cases. A logistic regression procedure was performed to assess if the baseline and averaged values of the biomarkers during the illness were predictive of survival. Associations of the biomarkers were tested by Spearman's rank correlation coefficient. A fitted logistic regression model was used to construct a probability of survival curve based on protein C levels and antithrombin levels. A probability <0.05 was considered statistically significant.

Results

Samples from 30 patients with suspected acute severe melioidosis were analyzed in this study. *B. pseudomallei* was subsequently isolated from clinical samples from 28 patients; two patients were culture-negative and thus not proven to have melioidosis. A large percentage of the patients were bacteremic, and the lung was the common site of focal infection. The mortality rate, measured as in-hospital mortality or when the patient was taken home moribund, was 40% (12/30). The demographics and baseline characteristics of survivors and fatal cases are presented in Table 1. The age and gender of survivors and fatal cases were comparable. Fatal cases had a higher mean APACHE II (Acute Physiology and Chronic Health Evaluation) score than survivors. Diabetes mellitus was a preexisting illness in 12/30 (40%) of the patients studied. A similar percentage of diabetics was found in the survivor and nonsurvivor groups. All the deaths occurred in the patients with bacteremia and in the population with pneumonia without bacteremia. Similar numbers of survivors and fatal cases suffered acute renal failure, but shock and liver function test abnormalities occurred more frequently in the fatal cases.

Protein C and protein S antigen levels, plus antithrombin activities, for fatal cases and survivors

Table 1 Demographics and baseline characteristics of study patients.

	Survivors (%) (n = 18)	Fatal cases (%) (n = 12)	Total (%) (n = 30)
Mean APACHE II score (range)	12 (0—19)	22 (17—30)	16 (0—30)
Mean age in years (range)	52 (28—74)	53 (32—82)	53 (28—82)
Gender			
Males	12 (67)	7 (58)	19 (63)
Females	6 (33)	5 (42)	11 (37)
Preexisting illness			
Diabetes mellitus	7 (39)	5 (42)	12 (40)
Chronic renal disease	1 (5.5)	1 (8)	2 (7)
Site of infection			
Bacteremia (with or without primary site)	5 (28)	9 (75)	14 (47)
Lung	6 (33)	3 (25)	9 (30)
Soft tissue	3 (17)	0	3 (10)
Spleen	1 (5.5)	0	1 (3)
Eye	1 (5.5)	0	1 (3)
Unconfirmed	2 (11)	0	2 (7)
Organ failure			
Shock	3 (17)	8 (67)	11 (37)
Acute renal failure	5 (28)	4 (33)	9 (30)
Liver function test abnormalities	8 (44)	10 (83)	18 (60)

Table 2 Comparison of baseline and average endothelial modulator markers in survivors and fatal cases (logistic regression).

Assay	Outcome	Number	Baseline mean ± SD	p Value	Average mean ± SD	p Value
Protein C (% antigen)	Died	12	43.4 ± 18	0.009	39.7 ± 13.8	0.004
	Survived	18	74.6 ± 27.8		80.7 ± 26.9	
Protein S (% antigen)	Died	12	65.7 ± 17.9	0.01	61.8 ± 16.1	0.006
	Survived	18	89.0 ± 18.6		87.2 ± 17.4	
Antithrombin (% activity)	Died	12	71.0 ± 20	0.02	63.5 ± 15.4	0.005
	Survived	17	90.4 ± 28.0		94.8 ± 25.6	

are shown in Table 2. Protein C antigen levels and antithrombin activities for fatal cases and survivors are shown in Figures 1 and 2 respectively. The mean baseline protein C antigen levels, protein S levels and antithrombin activity levels for all cases were 62.1% (standard deviation (SD) 28.6%), 79.7% (SD 21.5%), and 82.4% (SD 26.5%), respectively. Baseline values of protein C, protein S, and antithrombin were predictive of poor outcome, with significantly lower levels found in fatal cases than in survivors (Table 2; $p = 0.009$, 0.01 and 0.02, respectively). When values of these parameters were averaged for patients during the course of the illness, low protein C antigen levels, protein S antigen levels, and antithrombin activities continued to be predictive of a poor outcome (Table 2). On average, the levels of protein C, protein S and antithrombin were lower in the bacteremic patients compared with patients with a focal site of infection without bacteremia (data not shown).

The median plasma IL-6 concentration was 240.2 pg/mL (range 14.6—745 000 pg/mL, interquartile (IQ) range 64.9—1646.5 pg/mL). Both protein C and antithrombin values but not protein S were correlated inversely with IL-6 levels (protein C: Spearman correlation coefficient −0.74, $p = 0.0001$; antithrombin: Spearman correlation

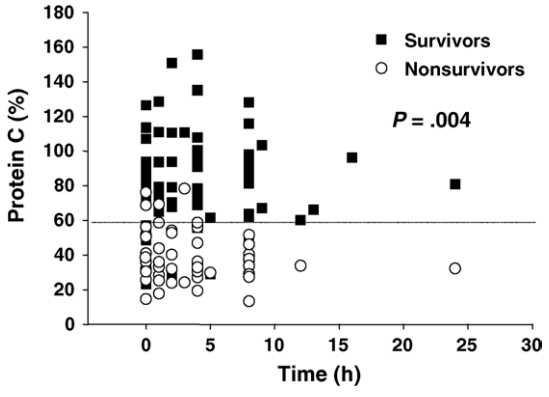

Figure 1 Protein C antigen levels over time in melioidosis patients, survivors and nonsurvivors. The dashed line represents the approximate level of protein C (60%) demarcating survivors from nonsurvivors.

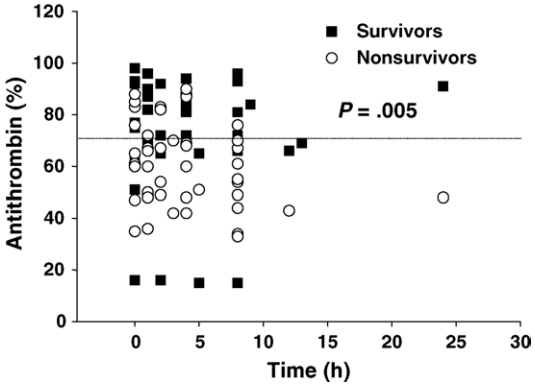

Figure 2 Antithrombin levels over time in melioidosis patients, survivors and nonsurvivors. The dashed line represents the approximate level of antithrombin (70%) demarcating survivors from nonsurvivors.

coefficient −0.68, $p = 0.0001$; protein S: Spearman correlation coefficient −0.32, $p = 0.077$). Baseline D-dimer levels were significantly higher in fatal cases than survivors (12.50 ± 11.1 μg/mL and 5.3 ± 10.3 μg/mL, respectively, $p = 0.004$ by ANOVA). Both protein C antigen and antithrombin activities were correlated inversely with D-dimer levels ($p = 0.005$ and 0.0065, respectively). Protein C and antithrombin levels were also highly correlated in these patients (R = rho = Pearson correlation coefficient = 0.82, $p = 0.0001$).

Discussion

Acute septicemic melioidosis is a homogenous form of Gram-negative sepsis, with a mortality of 35—45%. A study of proinflammatory cytokine levels in patients with melioidosis demonstrated that both baseline and peak TNF-alpha levels were inversely correlated with time to death. IL-6 concentrations have prognostic value.[9] Autopsy findings in patients who died from acute septicemic melioidosis have demonstrated fibrin clots as part of the pathology seen with the disease. Atisook and Panyathanya reported clinicopathologic findings from six autopsies performed on patients who died from acute septicemic melioidosis.[20] The findings in these patients included not only the diffuse abscesses typical of this infection but also coagulative tissue necrosis, petechial hemorrhages in the skin, lungs, and pericardium, and fibrin thrombi in the lungs, kidney, and adrenals, which are indicative of disseminated intravascular coagulation. These data taken together suggest interplay between inflammation and coagulation in the pathology of this form of sepsis.

Our study investigated systemic levels of three naturally occurring endothelial modulators as predictors of mortality in patients with Gram-negative sepsis. Depletion of protein C, protein S, and antithrombin at baseline was predictive of poor outcome. These findings have not been described previously in melioidosis. Average baseline levels of protein C were lower than were protein S and antithrombin levels in the population studied. Low levels of protein C, antithrombin, and protein S continued to be predictive of poor outcome throughout the illness. For protein C, a demarcation level of 60% of normal separated the survivors from fatal cases (Figure 1). For antithrombin, this cut-off is less clear but is just above 70% of normal (Figure 2). These data, taken together, suggest that a certain critical level of endothelial modulators is needed to prevent a vicious circle of inflammation and coagulation, which can lead to mortality from sepsis.

Low protein C and antithrombin levels were inversely correlated with the global marker of inflammation, IL-6. This finding further implicates the role of proinflammatory cytokines in the loss of these endogenous endothelial modulators in sepsis. Patients who died from melioidosis were found to have significantly higher levels of the fibrin degradation product D-dimer. Deficiencies in protein C and antithrombin were associated with higher levels of D-dimer, implying unchecked fibrin production and lysis, and suggesting fibrin deposition in the microcirculation as a mechanism that contributes to organ failure and death.

This study adds to the accumulating literature demonstrating the link between inflammation and coagulation in the septic patient. In a study of 43 adult patients who met established criteria for sepsis, admission values of antithrombin were significantly higher in survivors than in fatal cases (76%, range 65—87, and 64%, range 49—71) and inversely correlated with TNF-alpha levels.[21] Lorente et al. found that antithrombin and protein C levels, but not protein S levels, were significantly different in survivors and fatal cases of septic shock.[22] Similar findings in this population were observed by Fourrier et al., however, protein C and antithrombin levels that best predicted a poor outcome were lower than in our study (<40% and <50%, respectively).[23] These studies observed normal levels of free protein S that are in contrast to our findings. In summary, the literature in the heterogeneous group with sepsis is in agreement with our data in suggesting that antithrombin and protein C levels are predictive of patient outcome in sepsis.

Data are also available on the predictive value of endothelial modulator levels in homogeneous populations with specific infectious diseases and syndromes. Purpura fulminans is a syndrome in which low levels of endothelial modulators such as protein C are described. Infants born with homozygous protein C deficiency suffer life-threa-

tening purpura fulminans in the first few days of life.[24] In a study of 40 children with infectious purpura fulminans, antithrombin, protein C, and protein S were all significantly lower in fatal cases than in survivors ($p < 0.005$), with protein C the most depleted factor.[25] In 39 patients with systemic meningococcal disease, Brandtzaeg et al. found that the fatal cases were unable to achieve protein C levels three fourths of 55% or antithrombin levels three fourths of 60%.[26] In a study of 35 patients with meningococcal septic shock, antithrombin and protein C levels were significantly lower in fatal cases than survivors, with a protein C level <10% the best predictor of a fatal outcome. Interestingly, the level of free protein S did not differ significantly between survivors and fatal cases and the C4b-binding protein levels were not exceedingly high.[27]

Our data in melioidosis confirm the interplay of inflammation and coagulation in sepsis. Consumption of endothelial modulators is a key feature of this process. Protein C is the modulator most profoundly affected, followed by antithrombin and less so by protein S. The lowest levels of these endogenous anticoagulants were found in the bacteremic population, a similar finding to that seen in a heterogeneous population with severe sepsis.[28]

A testable hypothesis that arises from the accumulated data is that therapy with protein C and/or activated protein C and/or antithrombin could reduce mortality in melioidosis. It is possible to construct a fitted probability of survival curve from our data on protein C levels and antithrombin levels in melioidosis illustrating this hypothesis (Figures 3 and 4). Protein S therapy is a less attractive target because patients generally have adequate free protein S levels during the course of sepsis.[29] Furthermore, free protein S is in large molar excess over protein C or activated protein C levels.

Such hypotheses have been evaluated in two large Phase III mortality trials in patients with severe sepsis caused by a diverse array of organisms. A trial of antithrombin compared with placebo in patients with severe sepsis revealed no difference in 28-day all-cause mortality rates (relative risk = 1.004).[30] However, a very large Phase III trial comparing pharmacologic doses (24 μg/kg/h for 96 hours) of recombinant human activated protein C (drotrecogin alfa (activated)) with placebo in patients with severe sepsis demonstrated a 6.1% absolute reduction in, and 19.4% relative risk reduction in, the 28-day all-cause mortality in the treatment arm ($p = 0.005$).[31] It would be of considerable value, therefore, to conduct such a sepsis trial in disease caused by a single organism, such as melioidosis or meningococcal sepsis.

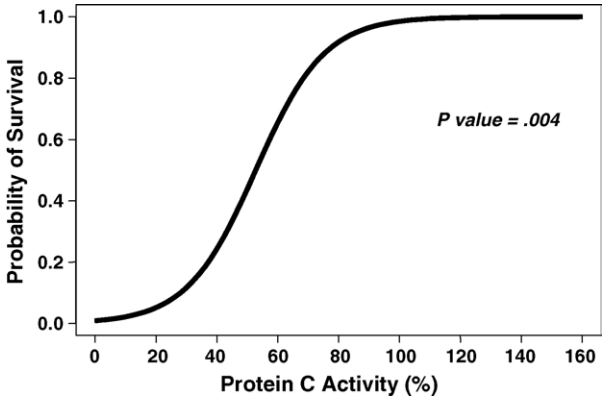

Figure 3 Predicted probability of survival in melioidosis patients. From logistic model for patients' average protein C antigen level predicting outcome ($n = 30$).

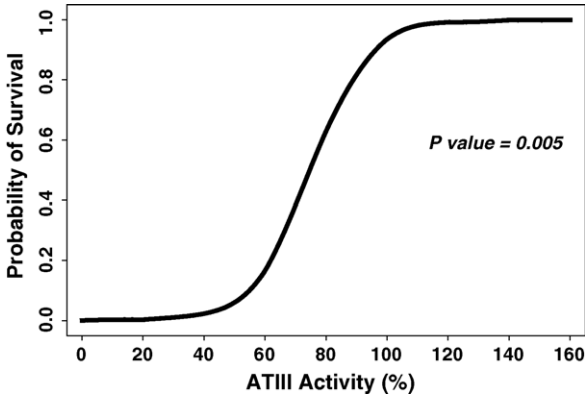

Figure 4 Predicted probability of survival in melioidosis patients. From logistic model for patients' average antithrombin level predicting outcome ($n = 30$).

The results reported here demonstrate the central role of inflammation and coagulopathy in the pathophysiology of severe sepsis caused by *B. pseudomallei* and the importance of activated protein C as a mediator of this process. If the substantial mortality still seen in acute melioidosis is to be reduced, clinical trials of agents such as activated protein C or antithrombin replacement therapy must be conducted soon.

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Conflict of interest: B. Utterback and S. B. Yan are employees and shareholders of Eli Lilly & Co., the manufacturer of recombinant human activated

protein C. J. Helterbrand is a former employee of Eli Lilly & Co. C. J. Fisher and S. P. LaRosa are former employees and former shareholders of Eli Lilly & Co. S Opal, A. J. H. Simpson, N. J. White and W. Chaowagul have no conflict of interest to declare.

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